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STORAGE ROT OF CARROTS INCITED BY A SCLEROTINIA-CANDIDA COMPLEX

by

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ABSTRACT

Cup rot of carrots was reproduced in closed polyethylene bags by inoculating with a combination of two fungi, Sclerotinia sclerotiorum and Candida krusei. The sequential appearance of S. sclerotiorum as the primary incitant of the disease, followed by C. krusei, was influenced by a set of conditions peculiar to the closed storage environment.

Craters ranging in diameter from 5 to 20 mm were produced on carrot tissue stored at 10° C for 29 days. A longer storage period was required to produce cup rot at 0° and 5° C, and at all temperatures cratering was most severe at tip-end inoculation sites. Bruised tissue, high humidity in storage bags, and a moist carrot surface, were prerequisites to development of cup rot.

Carrots were first invaded by S. sclerotiorum, and through its enzymic action host tissue was macerated and cell contents were released. However, carbon dioxide concentration in closed bags increased to over 20 percent while oxygen decreased to less than 2 percent with the result that growth of the pathogen was inhibited. These changes favoured development of the saprophyte, C. krusei, and the production of alcohol and related compounds further assisted in the killing of S. sclerotiorum. An increase in acidity of host tissue during initial penetration by S. sclerotiorum also favoured invasion of diseased tissue by the yeast. Growth of C. krusei, however, was limited to the area of tissue already damaged. Sound

tissue bordering the region affected by S. sclerotiorum provided an effective barrier to C. krusei and comprised the walls of the cup-shaped craters.

Tests on S. sclerotiorum in liquid culture using dextrose, ethyl alcohol, and carbon dioxide at different concentrations showed that growth of the fungus was stimulated by increasing the sugar content, and inhibited by increasing the amount of carbon dioxide and alcohol. The weight of mycelium continued increasing in concentrations up to 100 grams dextrose per litre Houston's medium. Further increases of sugar resulted in a growth decline. Ethyl alcohol at room temperature sharply inhibited growth of the fungus in Houston's medium at concentrations of 2.4, 2.6, 2.8, 3.0 and 3.2 percent, and completely suppressed growth at 3.4 percent. Carbon dioxide at 13 percent completely suppressed growth of S. sclerotiorum in Houston's medium at room temperature. Growth inhibition was sharp and continuous at concentrations greater than 1 percent. Stimulation of growth at 4 to 8 percent concentration of carbon dioxide occurred only in instances where inoculum discs were obtained from mycelium grown continuously on malt-yeast agar medium.

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INTRODUCTION

Several areas of North America and parts of northern Europe produce large acreages of carrots for fresh-market consumption and for winter storage. Most of the late crop carrots must be held for various periods of time in either common or cold storages. The nature of the fleshy storage tissue of carrots makes them vulnerable to attack by fungi and bacteria. The spoilage resulting from these attacks is a definite economic factor in both production and processing.

Alberta is well situated for the production of cool season vegetables such as carrots. The generally cool growing periods, free from extremes of temperature and moisture, provide the necessary conditions for crop growth. However, long winters combined with the relatively short growing season call for extended storage periods lasting as long as seven or eight months. During this time it is essential that storage temperature be kept low and humidity, in the case of carrots, very high. To meet this latter requirement an attempt was made to store newly-washed carrots, while wet, in sealed, heavy duty, fifty-pound polyethylene bags.

After three months of storage a type of rot occurred, as yet undescribed in the literature. Carrots within sealed bags stored at approximately 6° C developed cup-shaped craters (Fig. 1). Early symptoms of their development were discoloration and softening of tissue in a section about an inch wide extending approximately halfway around the circumference of the

Fig. 1. Symptoms of cup rot of carrots stored in sealed polyethylene bags.

carrot. This was followed by further tissue discoloration in the affected area until it became dark brown or almost black (Fig. 2). The centre of the affected area caved inward and the damaged surface tissue took on a glistening, slimy appearance, and was eventually covered by a thick creamy-white ooze (Fig. 3). Boundaries of the developing crater were distinct and firm. Finally, tissue collapsed, leaving cup-like craters extending to the core of the carrot. A distinct ferment was evident when the bags were opened. Preliminary investigations indicated that Sclerotinia sclerotiorum (Lib.) de Bary was initially associated with the rot. This association was eventually obscured by the yeast, Candida krusei, which frequently over-grew the entire affected area.

The purpose of this investigation was twofold: (1) to determine whether the Sclerotinia-Candida complex was primarily responsible for cup rot of carrots by reproducing the condition in the laboratory and, (2) to determine some of the physiological factors involved in the development of this rot, and consequently to show reasons for the successive appearance of the two fungi.

Fig. 2. Symptoms of early stages of cup rot of carrots.

Fig. 3. Symptoms of advanced stages of cup rot of carrots. Candida krusei has overgrown the diseased tissue.

GENERAL LITERATURE REVIEW

Carrots are subject to attacks by a wide range of bacteria and fungi. The control of these microorganisms makes storage costly and difficult. Among bacterial rots, Erwinia carotovora, which causes slimy soft-rot, is most common on carrots. The rot is characterized by tissue becoming water-soaked and collapsing into a soft, watery, slimy mass. The epidermis usually remains intact. This rot can be found in most common and pit storages, particularly when the storage period is extended into the spring months. It is found less frequently in cold storages, presumably because the temperatures are maintained below or close to the minimum for growth of the pathogen (56).

Among fungal rots of major importance, S. sclerotiorum, Botrytis cinerea, and Rhizoctonia carotae are the three principal pathogens affecting carrots in cold storage in most parts of the northern United States and in Canada. As a general rule losses caused by Botrytis rot are not great, except in blocks of carrots stored for two to three months or longer. Lesions caused by this pathogen are found most commonly either on the crown or on the tip. The diseased cells do not separate; thus the affected tissues are almost leathery in advanced stages of decay. The surface of the lesions usually becomes covered with the characteristic grayish-brown conidiophores and conidia.

Rhizoctonia crater rot may reach epiphytotic proportions and cause heavy losses where the relative humidities are high. Its presence has caused heavy losses (56) because the causal

organism can flourish at temperatures maintained in cold storages. Crater rot appears as pits on the roots under small, whitish hyphal knots. The pits enlarge into sunken craters lined with a white flocculent mycelium. Under high humidity the fungus spreads rapidly from these craters until the entire root may be covered with the white, cottony mycelium. The disease at this stage appears somewhat similar to that of the rot caused by S. sclerotiorum, but is distinguishable from it by a looser, weft-like mycelium and the absence of sclerotia.

The amount of Sclerotinia decay found in storage is determined by the extent of infection in the field and infestation of storage containers. Prevalence of the disease is in direct proportion to the number of years carrots are grown in the same soil (48). The rapidity with which storage tissue is demolished is partly due to the initial impetus provided the pathogen by high humidities necessary when storing carrots and, in part, to the fact that hydrosis of the infected tissue supplies additional moisture for subsequent development and spread. In spite of the use of low temperature storage in transit, the disease continues to be of major economic importance. Invasion of carrots has been noted at 0° C (37, 56). A detailed account of the symptoms of the rot caused by S. sclerotiorum will be given in a later section of this report.

Yeasts, on the other hand, although world-wide in distribution, only occasionally are reported as being associated with plant pathogenic organisms causing rot of carrots (47).

GENERAL MATERIALS AND METHODS

Carrot Varieties

Two carrot varieties, Imperator 11 and Empress, were used. Both are mid-season crops grown widely in southern Alberta. They were grown on prairie soils which had been irrigated and fertilized with ammonium phosphate (16-20-0) at the rate of 200 pounds per acre. Mechanical harvesters lifted the plants and twisted off the tops before depositing the roots in waiting conveyances.

Storage Containers

Storage containers were of low density type polyethylene manufactured by Canadian Industries Limited. Thickness of bags was 6 mil with a gas diffusion rate at 21° C and relative humidity zero of 483 c.c. carbon dioxide per 100 square inches bag surface area per 24 hours at 1 atmosphere pressure. Oxygen diffusion rating was 92 c.c. oxygen per square inch surface per 24 hours at 1 atmosphere pressure.

Fungus Isolates

One isolate of *S. sclerotiorum* originating with infested carrots from the Brooks, Alberta, area was used. The species is classified among the Discomycetes or cup fungi as they are known. As a member of the inoperculate group of Discomycetes, the asci have no regular pore, but burst open irregularly at the tip, releasing their spores (73). The Sclerotineaceae comprise one of the largest of the 14 families of this group. From the economic standpoint, it is the most important one. The mycelium

of S. sclerotiorum is hyaline and branched. The sclerotia range in diameter from 2.5 to 6 mm. No definite period of dormancy appears to be necessary for the production of apothecia. The stipe arises from the sclerotium and gives rise to a funnel-shaped cup from which ascospores are discharged in great numbers. The ascospores are hyaline, continuous and ovate. The average size of asci and ascospores respectively is 8.2 to 10.2 u by 125.4 to 160.4 u and 5.9 to 7.3 u by 11.7 to 5.1 u. No true conidia are produced. Microconidia are formed, usually as the available food supply is on the decline. They are formed in chains at the tips of short lateral branches of the vegetative mycelium. They apparently do not function in the infection and in the dissemination of the fungus (73).

The yeast, C. krusei, was obtained from the same infested carrots which furnished Sclerotinia inoculum. Identification of C. krusei was provided by the Centraalbureau Voor Schimmelcultures, Delft, Holland. The fungus is an asporogenous, mycelial-forming, fermenting yeast. The surface growth on agar is largely composed of budding cells. Its most outstanding feature is the development of true hyphae with partition walls (38). Budding cells or blastospores are formed in abundance on the filaments. On nitrogen-deficient media chlamydospores frequently develop. In liquid media growth is in the form of a surface film. Unlike some species of Candida, C. krusei ferments glucose vigorously. It also assimilates citric acid, ethanol, glycerol, lactic acid, succinic acid, sucrose, xylose and potassium-D-gluconate (46).

Both fungi were carried on artificial media, C. krusei on potato sucrose agar and S. sclerotiorum on malt yeast agar by mass transfers. With certain exceptions S. sclerotiorum maintained its original vigorous growth habit and pathogenicity. Mycelium or sliced sclerotia grown on liquid medium consistently grew well when transferred to liquid or solid media. On the other hand, mycelium or sliced sclerotia grown and transferred several times on solid media eventually grew poorly, or not at all, when placed in liquid shake culture or in slanted tubes of malt yeast agar. The yeast grew well throughout.

PART I. INITIATION AND DEVELOPMENT OF CUP ROT

LITERATURE REVIEW

Sclerotinia sclerotiorum, the incitant of watery rot of carrots, survives from season to season as active mycelium in either living or dead plants, but especially in the form of the hard black sclerotia. In warmer climates these sclerotia are formed sparingly and do not germinate before being subjected to low temperature. Each sclerotium produces one to many stalks with a small flesh-colored, flat, or saucer-shaped tip. On the upper surface is a layer of microscopic sacs each with eight spores. Although moist winds blow these spores from one field to another, the principal means of fungus dissemination is the shipment of diseased plant parts or as sclerotia mixed with seeds. If the sclerotia are dry, they may remain alive a number of years. When wet they decay readily and may be destroyed in less than a year. The ascospores live only a few hours or until they become dry. When they land on susceptible plant parts, infection can occur within 2 days and symptoms can be pronounced in 4 days. No conidial stage of the fungus has been found. The fungus requires abundant moisture, thriving best where there are continuous rains, or under sprinkler irrigation (11).

On carrots or other infected roots the presence of the cottony mycelium and the black sclerotia are characteristic signs of the disease (Fig. 4). The invaded tissues are somewhat darker in color than are the healthy ones. As the decay continues they become soft and watery but without sliminess.

Fig. 4. Symptoms of Sclerotinia watery rot of a carrot.

Roots may be covered in part, or completely, by the mycelial mass. When the fungus growth begins at a central point and progresses outward in packages that are being stored or shipped, the disease is termed nesting or nestiness.

Several workers have investigated the histological aspects of S. sclerotiorum on susceptible host tissue (5, 18, 54). DeBary (4) concluded that the fungus penetrated the cuticle by softening and dissolving the membranes. He postulated that a toxic material produced by appressoria diffused into the host tissue, killing some of the cells and releasing nutrients which diffused back into the infection area. Boyle (5) observed the following sequence to have occurred. First the hyphae became enveloped in a thick, mucilaginous sheath which caused the mycelium to adhere to the surface of the host. Contact stimulus caused formation of large thick-walled appressoria. Small infection hyphae emerged from the thickened walls with a pressure which caused indentation of the host cuticle. Mechanical rupturing of the cuticle occurred and infection hyphae swelled into vesicles in the epidermal cells. Simultaneously, chemical alterations of cell walls, chloroplasts, and cell contents occurred. Ahead of the advancing hyphae the cells died due to fungal enzyme action, or to damaged host cell products. Boyle found no evidence to support DeBary's contention that death of the underlying cells took place before rupturing and penetration of the cuticle.

Recently Brown (8), in a review of the literature dealing

with toxins and cell-wall dissolving enzymes, examined the reports of numerous workers. In his view, there was little doubt that the macerating principle was an enzyme or enzymes. The action was that of enzymes of the pectolytic type. The depolymerase (DP) group aided in the hydrolysis of the pectate chains of the middle lamella to form polygalacturonides (32, 61, 74). Pectin methyl esterase (PME) acted on the methyl groups of the pectin chains in the secondary cell walls (73). The lethal principle seemed to be intimately tied up with the macerating action on cell walls, since death of cell protoplasts stopped when the macerating extracts were artificially inactivated (20). That pathogenicity was closely related to enzyme activity has been demonstrated by Cole (12) who studied the action of S. fructigena on pome fruits. Apparently the sequence of events was that while the fungal hyphae grew through the tissue, macerating and killing cells, oxidized phenolic compounds that tended to inactivate the macerating enzyme were formed. This action, however, did not come into play until the fungal enzyme had macerated and killed the host cells at the margin of the lesion. Thus the progress of invasion was not impeded. The state of turgor of host cells seemed to be of considerable importance in determining the extent of attack by the invading fungus and the amount of tissue which could be macerated by enzyme preparations extracted from invaded tissue.

Several reports deal with the action of a group of organisms exhibiting similar properties (19, 22, 36). The results

inferred that the macerating system of the invading organism was sensitive to some factor present in sub-turgid tissue. Brown (8) suggested that no freely available water was present in walls of sub-turgid cells. Unless a great deal of water accompanied the invading organism, surface water was rapidly passed inward from outer cell layers to deeper cells leaving the surface layer and the fungus relatively dry. Growth of the fungus ceased and little or no attack took place. Mukula (48) reported greatly delayed action of S. sclerotiorum on carrots, the surfaces of which were pre-dried before storage. These results were supported by the work of Lauritzen (37) who tested the effects of the fungus on wet and dry carrots.

The value of carbon dioxide in delaying rotting by fungi was suggested by Kidd and West (34) to be partly due to a direct effect on the fungus and, in part, to physiological effects on the host. Thornton (66, 67) showed that increased carbon dioxide tended to make the sap in living carrot cells more alkaline, and this was decidedly unfavourable to the development of S. sclerotiorum. Expressed cell sap, however, was made more acid. Macerating action of the enzymes of the fungus on carrot tissue was at its peak at about pH 4 (18).

Candida krusei is rather widespread in nature, particularly in orchard soils. Morphologically, its most outstanding feature is its ability, under certain conditions, to develop mycelia with true hyphae having partition walls. Little is known of its mode of action as a spoilage organism. As in other yeasts, its chief physiological peculiarity is its ability

to effect the alcohol fermentation of sugars in the absence of air. It is mentioned several times as occurring on spoiled foods containing lactic acid, such as pickle brine, milk, fruit, and sauerkraut (40). The organism evidently thrives at a very low pH (29) and, like most yeasts, is very resistant to low temperature. C. krusei is not one of the few yeasts which have the ability to hydrolyze pectin (38). It appears to be fairly closely related to another false yeast, Geotrichum candidum, reported to cause sour rot of carrots (75). This organism, also, is frequently associated with molding of foods containing lactic acid, and causes a white skin-like growth to form on some food preparations. During the process of glycolytic breakdown of pyruvate the addition of yeast tends to swing the reaction away from the formation of lactic acid, and favours the production of ethyl alcohol (1, 27, 30). C. krusei, however, seems to occur mainly in the presence of lactic acid and little alcohol.

MATERIALS AND METHODS

Packages and Packing

The carrot material for investigations on pathology was selected from bulk storage of the same varieties, grown on similar soil, as the host material in which cup rot was originally discovered. Polyethylene bags of 6 mil thickness were selected for their relative impermeability to carbon dioxide and oxygen; and were similar in thickness to those in which carrot rot occurred in storage. Bags were shortened to a size which would conveniently accommodate whole carrots without excess air space

(Fig. 5). Each bag was sealed using a type of fibre friction tape, after squeezing out most of the excess air. An outer bag, sealed in the same manner, provided additional protection against damage and leakage.

Washing and Disinfestation

The carrots had originally been washed in a tumbler-type slatted-wood washer with a central metal drive shaft. During washing carrots were observed to strike, sometimes forcibly, the metal drive shaft. Carrots for inoculation tests were removed from the 50-pound perforated polyethylene bag in which they were stored at 0° C. Several tests for surface disinfestation, using propylene oxide as reported by Hanson and Snyder (26), were made. However, surface injury occurred following a 3-hour exposure to 0.5 c.c. per litre of propylene oxide. At 0.3 c.c. per litre for 3 hours no injury resulted but, at this concentration, cells of C. krusei were not killed. Similarly, a 5-minute immersion of a 5 percent household bleach solution was ineffective. A 10-minute immersion in 10 percent bleach provided the desired effect. This was the method selected for treating carrots to be inoculated.

Inoculum and Inoculation

Inoculum of S. sclerotiorum was prepared by growing the fungus in Houston's liquid medium until sclerotia formed. These sclerotia were used as inoculum, and were supplemented by mycelia mixed with MYA from culture slants. Yeast inoculum was produced on PSA in slanted culture tubes.

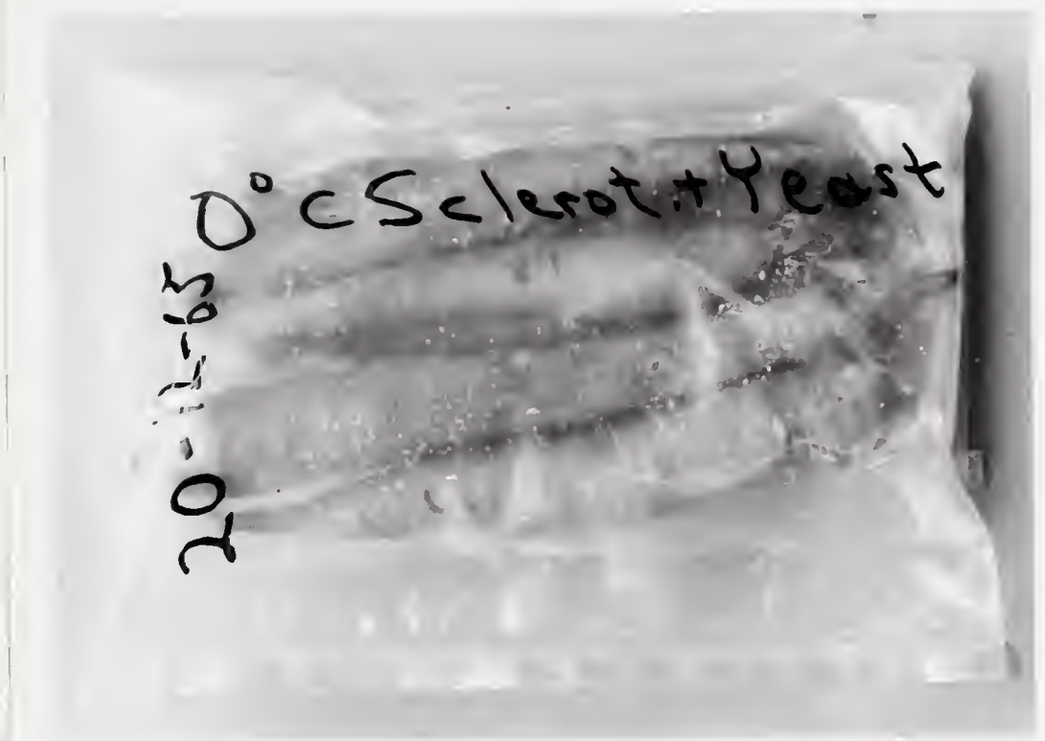

Fig. 5. The outer and inner heavy-duty polyethylene bags used in the laboratory for studies on cup rot of carrots.

The following methods of inoculum placement were used:

(1) Beneath 5 m.m. diameter cork-borer plugs, 5 m.m. deep, no covering.

(2) Beneath 5 m.m. diameter cork-borer plugs, covered with MYA or PSA.

(3) Pressed into scalpel cuts, no covering.

(4) Pressed into scalpel cuts, covered with MYA or PSA.

Cork borer plugs as described by Lauritzen (37), covered or uncovered, gave inconsistent results. Carrot tissue tended to dry out at the base of the well. Tissue which was sliced with parallel scalpel cuts and covered with solid media remained moist. This was the method employed. In one of the tests carrots were thoroughly washed in running tap water and laid out on paper to surface dry. They were then replaced in the polyethylene bag, stored overnight at 5° C., and immersed in a 5 percent bleach solution for 5 minutes the following morning. Following rinsing in running tap water, the carrots were again set out on paper to dry. Scalpel-cut type of inoculation followed and the carrots were placed in bags. They were sprayed lightly with water and the bags were sealed. This was the surface pre-drying treatment.

Bruising prior to inoculation was accomplished by steady manual pressure over the inoculation site using a 12 m.m. round aluminum rod. Pressure was released when a portion of the underlying tissue flattened. Each carrot was dipped in distilled water prior to inoculation. Using inoculation placement method #4, a mixture of chopped sclerotia and mycelia were pressed into scalpel cuts. When S. sclerotiorum was used alone MYA was

incorporated into the inoculated cut surface tissue. When C. krusei was the inoculum, PSA was used. Both media were incorporated at the inoculation site for combined inoculum.

Four bags of 8 carrots each were used for each storage temperature. Carrots were carefully selected for uniformity, smoothness and size. Four bruised and 4 non-bruised carrots were placed in a bag. One bag contained carrots inoculated with S. sclerotiorum and C. krusei, one S. sclerotiorum alone, one C. krusei alone, and one bag contained control carrots. Prior to sealing each bag the interior was atomized with water to raise the relative humidity.

Carrots were periodically checked for cratering by visual methods and by feeling for soft spots without opening storage bags. Considerable care was necessary in handling the bags stored at low temperature, owing to the tendency of the sealing tape to crack open. When any treatment showed a noticeable breakdown in several carrots, the air in all the bags in that treatment was analyzed for quantities of carbon dioxide and oxygen. Immediately following gas analysis, disease development was rated and recorded.

Storage Temperature and Humidity

Inoculated material was stored in refrigerators at 0°, 5°, or 10° C. Preliminary trials, using wet-and-dry bulb hygrometers, indicated that moistened carrots maintained a relative humidity within the bags of over 95 percent at all temperatures. At 0° and 5° C condensation occurred within 2 - 3 days after bags were sealed.

Storage Atmosphere

The atmosphere inside all polyethylene bags for a given temperature treatment was sampled when carrots in any bag had cratered noticeably. Carbon dioxide and oxygen content of the inner sealed bag were measured with a Haldane-Henderson gas analyzer (Fig. 6). The closed system of the apparatus was flushed by drawing approximately 10 c.c. of room atmosphere which was scrubbed through KOH and through alkaline pyrogallol to remove carbon dioxide and oxygen respectively. A small rubber balloon from which the air had been excluded was then fastened over the air intake tube. The outer plastic bag from a package of carrots was removed and, 10 c.c. of atmosphere was withdrawn from the sealed inner bag, using a hypodermic syringe, and the gas was released through the syringe into the deflated balloon on the instrument. The bag perforation caused by the syringe needle was quickly sealed with adhesive tape. Duplicate readings were taken for each bag. Readings were accurate to 0.1 percent, similar to Brown's report (7) on use of a Haldane gas analyzer. For concentrations over 25 percent lack of available calibration of the instrument necessitated a dilution of the gas being measured. In such cases 1 c.c. of storage bag atmosphere was diluted in the syringe with 9 c.c. of room atmosphere. The ensuing mixture could then be read on the instrument, and the necessary compensating calculations were made.

Alcohol Analysis

Alcohol content of cratered carrot tissue was determined

Fig. 6. The Haldane-Henderson Gas Analysis apparatus used for measuring concentrations of carbon dioxide and oxygen in closed storage bags containing carrots.

using a Beckman GC-2 gas chromatograph (76). Tissue for analysis was prepared in the following manner:

(1) Weight of rotted tissue to be analyzed was recorded.

(2) The required volume of propyl acetate extraction fluid was determined.

(3) Normal propyl acetate was combined with the rotted carrot tissue.

(4) The propyl acetate extract containing alcohol from carrot tissue was injected into the gas chromatograph.

Analysis was usually done within a day or two after crater recording. In the interval carrots remained stored at low temperature. Bags were kept sealed to prevent possible evaporation of alcohol.

pH Measurements

Measurements of pH of carrot tissue were obtained, using a Beckman pH meter, after thoroughly macerating the tissue in a Waring blendor. Growth of S. sclerotiorum was readily removed by scraping the surface of MYA in culture plates. Mycelium was blended with distilled water prior to taking pH readings.

EXPERIMENTAL RESULTS

Inoculation Site and Bruising

Severity of cup rot was greater on the tip end of inoculated carrots than at either the middle or the hypocotyl (Fig. 7). In order of increasing severity of cratering, those of 5 to 10 m.m. diameter are listed as 1, those which were 10

Fig. 7. Severity of cup rot as influenced by location of infection court on carrots.

to 15 m.m. in diameter as 2; and a 15 to 20 m.m. diameter crater as 3 (Table 1).

Table 1. Effect of bruising carrots at 3 different sites prior to inoculation with a mixture of S. sclerotiorum and C. krusei on the development of cup rot in sealed polyethylene bags stored at 2 different temperatures.

Temperature	Treatment	Extent* of rot at following sites		
		Tip End	Center Tap Root	Hypocotyl
0° C	Bruised	2	1	1
	Not Bruised	0	0	0
10° C	Bruised	3	2	2
	Not Bruised	0	0	0

*0, no crater.
1, slight crater.
2, moderate crater.
3, deep crater.

Bruising was an essential factor in inducing the formation of cup rot since rot did not occur in any treatment in the absence of bruising of host tissue before inoculation. Host tissue bruised prior to inoculation cupped readily when the 2 inocula were combined. Cupping also tended to be more severe at the higher storage temperature.

Effect of Temperature

Cupping invariably occurred first in roots stored at the highest temperature (Table 2). Average length of time for noticeable cratering to occur, in four trials at 10° C,

Table 2. Effect of temperature on the time required to produce cup rot of carrots inoculated with a mixture of S. sclerotiorum and C. krusei and stored in sealed polyethylene bags.

Temperature	Number of days required to produce cup rot			
	Trial 1	Trial 2	Trial 3	Trial 4
0° C	44	41	76	57
5° C	36	41	67	75
10° C	28	21	34	34

was 29 days. On two occasions roots stored at 5° C cratered in 36 and 41 days. Those stored at 0° C, on the average, developed craters in 52 days. Not only did cratering occur more rapidly at the higher storage temperatures but the craters were wider and deeper than those occurring on carrots stored at 0° C.

Data in Table 2 show that cup rot may be induced artificially but the variability of results indicates that there are some uncontrolled factors which have an influence on the rapidity of the development of this rot. There is, however, a trend to most rapid rotting at the higher temperature of 10° C, and at the lower temperatures of 0° and 5° C the rot proceeds more slowly but at approximately the same rate in each.

Effect of Surface Drying

When carrots were pre-dried before inoculation practically no moisture condensation occurred in the bags during the storage period. The surface of the carrots appeared dry. Cratering did not occur after 75 days storage at 5° C (Table 3).

Table 3. Effect of surface drying of carrots prior to inoculation with a mixture of S. sclerotiorum and C. krusei on the development of cup rot in sealed polyethylene bags stored at 5° C.

Treatment	Extent* of rot at following sites:		
	Tip End	Center Tap Root	Hypocotyl
Pre-dried	0	0	0
Moistened	2	1	1
Moistened, not inoculated	0	0	0

*0, no crater.
1, slight crater.
2, moderate crater.
3, deep crater.

Carrots which were not pre-dried cratered readily.

Changes in Storage Atmosphere

When carrots were inoculated with either one or a combination of two fungi and placed in storage, oxygen levels dropped considerably for all treatments. At the time of noticeable cratering, levels of less than 2 percent characterized those treatments stored at 0° and 10° C (Table 4). There was little difference in oxygen content depending on whether the fungi were used singly or in combination. The bags of checks, however, had a relatively high oxygen content of 13 percent.

Carbon dioxide achieved a higher level at 0° C than at 10° C, where one or the other fungus inoculum was used alone. When combined inoculum was used, the carbon dioxide content of storage bags was greater at the higher temperature. It increased only moderately in the bag of checks, reaching the 5 percent level.

Table 4. Quantities of carbon dioxide and oxygen in sealed polyethylene bags containing carrots inoculated with either one or a combination of two fungi and stored at two temperatures until cup rot developed fully in any bag within the treatment.

Treatment	Percent gases in bags inoculated with:							
	Sclerotinia		Candida		Both		None	
	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
0° C	39.0	0.5	35.0	0.5	21.0	1.0	5.0	13.0
10° C	28.0	2.0	24.0	2.0	24.0	2.0	-	-

Oxygen in the atmosphere inside bags surrounding pre-dried carrots remained at a high level throughout the storage period (Table 5). Carbon dioxide increased only to 1 percent of the bag atmosphere. By contrast, carbon dioxide around the moistened checks increased to 10 percent. Around the moistened inoculated carrots it increased to over 40 percent. Oxygen in the bag of moistened checks dropped to approximately half the concentration maintained in bags of pre-dried carrots. In bags of moistened, inoculated carrots, oxygen was almost completely exhausted.

Table 5. Effect of surface drying of carrots prior to inoculation with a mixture of *S. sclerotiorum* and *C. krusei* on quantities of carbon dioxide and oxygen in sealed polyethylene bags following storage for 75 days at 5° C.

Treatment	Percent of gases in sealed bags	
	CO_2	O_2
Pre-dried, inoculated	1.0	19.0
Moistened, inoculated	46.0	0.5
Moistened, not inoculated	10.0	11.0

Production of Alcohol and Related Compounds

Tissue decomposition and accumulation of yeast was obvious at inoculation sites involving a combination of the two fungi. Fermented cell fluids over-ran these sites (Fig. 8) and collected in pools on the inner surface of storage bags. Alcohol yield at 0° C was approximately three times the amount found in a bag stored at 5° C and ten times the amount of alcohol in a bag stored at 10° C (Table 6). When C. krusei was the only inoculum cratering was absent and alcohol yields were considerably lower at all temperatures. The results, involving S. sclerotiorum as the inoculum, indicate even lower yields of alcohol. Non-inoculated carrots under identical storage conditions had an alcohol content almost as great as that involving S. sclerotiorum alone. Less alcohol was measured at 5° and 10° C storage, in most treatments, than at 0° C.

Table 6. Quantities of ethyl alcohol in diseased tissue and of carbon dioxide in sealed bags containing carrots inoculated with either one or a combination of 2 fungi and stored at 3 different temperatures.

	Percent alcohol and carbon dioxide (w/w) (v/v)							
Temperature	Sclerotinia		Candida		Both		None (check)	
	Alcohol	CO_2	Alcohol	CO_2	Alcohol	CO_2	Alcohol	CO_2
0° C	0.53	28	0.85	24	3.54	24	0.35	5
5° C	0.90	44	0.80	50	1.10	40	-	10
10° C	0.20	-	0.22	-	0.32	-	-	-

In addition to ethyl alcohol, traces of acetone, methyl alcohol, and isopropyl alcohol were present in all treatments.

Fig. 8. Advanced stages of cup rot of carrots in a sealed polyethylene bag showing accumulation of Candida krusei and fermented host cell fluid.

Amounts of each substance ranged from a low but measurable trace to 0.03 percent. Non-inoculated carrots yielded smaller amounts of these substances. Lactic acid was found in trace amounts in carrot tissue where either C. krusei or S. sclerotiorum were used alone. A somewhat higher amount occurred in carrots inoculated with the two fungi.

Carbon dioxide varied only slightly between treatment (Table 6) for a given storage temperature. However, a noticeable difference occurred between temperatures, with 40 to 50 percent concentration at 5° C as compared with 24 to 28 percent at 0° C. An increase in carbon dioxide did not correspond to an increase in ethyl alcohol.

Fungal Development and Changes in pH

After cup rot appeared, sealed storage bags were opened and carrot tissue was examined with a microscope. C. krusei was abundant and in a state of active growth. The few strands of mycelium of S. sclerotiorum that were found appeared to be dead. Inoculated carrots were allowed to remain in the opened bags for several days at low temperature during which time no growth of S. sclerotiorum appeared.

The following are some pH measurements of carrot tissue and culture filtrates for conditions as stated:

	pH
(1) Normal healthy carrot tissue	6.3
(2) Normal healthy tissue after 4 weeks storage at 5° C	6.3

	pH
(3) Normal healthy tissue after 22 weeks storage at 5° C	6.0
(4) Tissue affected by severe Sclerotinia rot	3.0
(5) Houston's liquid culture medium	6.8
(6) Houston's filtrate after 6 days Sclerotinia growth	3.0
(7) Houston's filtrate after 8 weeks Sclerotinia growth	6.0
(8) Sclerotinia scrapings alone, after 3 days growth	5.4

Prolonged storage at low temperature did not noticeably lower the pH of carrot tissue. S. sclerotiorum caused a considerable lowering of pH of carrot tissue. It also, greatly lowered the pH of Houston's liquid culture medium.

DISCUSSION

Initial investigations involved fungal penetration and host reaction to S. sclerotiorum and C. krusei on commercial varieties of carrots. Moistened carrots developed cup rot under the combined effects of the two fungi. The importance of having plentiful surface moisture, in order to secure tissue penetration, was demonstrated through tests involving pre-dried carrots. Although they were exposed to room temperature and low humidity for only a short time, it was sufficient to result in a loss of moisture which could not be replaced by briefly dipping the carrots in water followed by spraying water over the carrots within bags prior to sealing. According to Brown (8), when water is applied to the surface of subturgid storage tissue it rapidly

moves inward to make up the water deficit of underlying tissue. Unless such tissue is injected or immersed for a long period in water the outermost layer of cells will remain dry. Under such circumstances free moisture will not collect on the carrot surface and a fungus such as S. sclerotiorum having a high moisture requirement will be unable to develop.

Where cup rot was reproduced, it was possible to protect inoculum and inoculation sites from drying out by restricting incisions to surface layers of tissue and by coating the area with an agar medium. These measures were successful, however, only if carrots were protected from surface drying during inoculation. Evidently moisture requirements of the fungus were met by these precautions for microscopic examination of cratered tissue showed the presence of mycelium of S. sclerotiorum. The strands were few, however, and almost obscured by the abundant growth of C. krusei.

Bruising tissue was a prerequisite to development of cup rot and may be related to the physical as well as the physiological requirements of both fungi. As previously mentioned, both grow best at a very low pH. Carrot tissue normally has a pH between 6.0 and 6.5. The living-cell sap becomes more alkaline under the influence of increased carbon dioxide. However, when cells are killed or disrupted under the influence of bruising or fungal penetration, the pH is lowered by increased carbon dioxide. The result is a more favourable environment for the development of both fungi. Bruising apparently results in contents from ruptured cells being a readily available food supply

for C. krusei. The extent to which S. sclerotiorum is able to penetrate bruised tissue, during the relatively short time before carbon dioxide inhibits it, governs the total supply of nutrients available to C. krusei and, therefore, the extent of cup rot which follows.

The deep-well placement of inoculum (37) was not as satisfactory as the method outlined above. In many instances the round plug of carrot tissue removed by the cork borer tended to shrivel when replaced in the well following inoculation. This frequently resulted in the plug dislodging and a general drying out of the tissue at the base of the well. Under these conditions the inoculum failed to develop. Covering the plug site with a smear of solid media only partly alleviated the problem.

In the portion of outline dealing with general materials and methods it was mentioned that sclerotia or mycelium grown in liquid culture grew better than that which was produced on MYA; also, in the results of inoculations, that it gave a better response in the production of cup rot than sclerotia or mycelium produced on MYA. The reason for these phenomena remained obscure but preliminary investigation suggested the possibility of a staling reaction. At any rate the use of mycelium and sclerotia grown on MYA for prolonged periods was a risky procedure and often led to unpredictable results.

The tapered end of inoculated carrots cupped more readily and produced larger cups than the centre region of the root or hypocotyl. Since the tapered end contains a higher proportion of young growing tissue, such a result might be predicted. In

addition, the effect of pressure applied to the rod used in bruising carrot tissue probably was greater toward the growing tip. The fact that noticeable cup rot appeared only in bruised tissue emphasizes the importance of bruising in attempts to reproduce the disease.

At 10° C both C. krusei and S. sclerotiorum are more active than at low temperatures and this may account, in part, for the appearance of larger craters at the higher temperature. Assuming that S. sclerotiorum, through its macerating action on host cell walls, is the initial invader, penetration of tissue would be wider and deeper at the temperature nearest optimum for the pathogen. Thus, at higher storage temperatures a greater potential for cratering may exist than at lower ones.

High carbon dioxide levels have been shown to be detrimental to the growth of several fungi (7). The inhibiting influence is greatly enhanced by a drop in temperature. Pathogens, such as S. sclerotiorum, found growing near the soil surface are retarded by lower carbon dioxide concentrations than are pathogens such as Fusarium oxysporum which are capable of growth in the lower soil depths (68). Development of S. sclerotiorum on carrot tissue in sealed bags would cease when carbon dioxide concentrations reached total inhibition levels. That total inhibition did actually occur was evident from the absence of the typical white mycelium which is associated with development of the fungus even at low temperature. Some penetration of carrot tissue likely occurred, however, for cup rot followed the use of joint inoculum, whereas, when C. krusei was used alone no cup

rot occurred.

A decrease of oxygen content in storage bag atmosphere (Table 5) may have been a contributing influence toward the inhibition of S. sclerotiorum. During the storage period oxygen levels around moistened, inoculated carrots dropped below 3 percent, the extinction point for carrot tissue (30). Below the extinction point the production of such retardation products as alcohol and carbon dioxide occurred. However, the indirect effect of oxygen depletion is less likely to have been restrictive to S. sclerotiorum than the increase of ethyl alcohol which was associated with the development of C. krusei. Wherever this fungus was not involved in inoculations, alcohol in carrot tissue was low. Readings were similar to those reported by workers (27, 31) studying alcohol in carrot tissue. A shortage of available sugars may account for the relatively low alcohol readings where C. krusei was the single inoculum. Also, the fungus makes its best development at a low pH (29). The availability of cell nutrients, and a lowering of pH depend on the presence and initial activity of S. sclerotiorum.

The formation of other products such as acetone, methyl alcohol, and isopropyl alcohol had been anticipated. Their presence in carrot tissue, under near anaerobic conditions has been reported (1, 27, 30). Apparently, in the absence of yeast, utilization of diphosphopyridine nucleotide-reduced (DPNH) in reduction of pyruvate results in an accumulation of lactic acid. In the presence of yeast, however, the reaction tends to swing more in a direction to form acetaldehyde and ethyl alcohol (3).

Utilization of alcohol by undamaged carrot tissue has been reported (15), and may account for the lower amounts found in tissue stored at 10° C than at 0° C.

From these observations it appears likely that the development of cup rot rather than the typical Sclerotinia soft rot was due to environmental factors and their effects on the two fungi involved.

PART II. PHYSIOLOGY OF SCLEROTINIA SCLEROTIORUM AND OF CANDIDA KRUSEI

LITERATURE REVIEW

Several workers have investigated the nutritional requirements of S. sclerotiorum and related species. Newton (50) observed no change in growth of the fungus when sugar or any constituent ion was omitted from the nutrient medium. The addition of sulfate, calcium, and magnesium ions increased weight of mycelium produced, while ammonia replacing nitrate as a source of nitrogen inhibited growth considerably. Demetriades (16) reported that peptone proved to be the best source of nitrogen, of a number of different substances tried. Such a nitrogen source, however, seriously reduced the virulence of the fungus (21); the better the nitrogen source for fungal growth, the poorer the fungal penetration of host tissue. Tanrikut and Vaughan's (65) results conflicted with Newton's, and both were investigated by Purdy and Grogan (55) who found a significant growth reduction in nutrient solutions lacking phosphorus, magnesium, and trace elements. There was little or no development in the absence of potassium, nitrogen, or carbon.

Saburova (58) obtained highest infection of S. sclerotiorum on commercial stored carrots grown under high nitrogen conditions. Lowest infection occurred on carrots grown with a high potassium fertilizer. Apparently, potassium reduced the hydrolysis process of complex organic compounds to simple compounds in infected roots. Shumilenko (60) obtained substantially the same results

for potassium and phosphorus. Mukula (48) suggested that these two elements increased the sucrose-glucose ratio thus increasing the keeping quality of carrots. His review of the results (2, 24, 35), where heavy nitrogen applications were made, suggested increased storage decay. Hasselbring (25), studying the carbohydrate transformation during storage found the principal changes to be a conversion of sucrose to reducing sugars, and a transformation of polysaccharides to simple sugars until an equilibrium was reached in about 10 weeks. Twenty-eight percent of the sucrose, which constituted up to 4 percent of carrot weight at harvest, was lost during this period at low temperature. Platenius (52) showed that, after the early sugar conversion period, this process was later reversed in storage.

S. sclerotiorum grew well on Houston's solution (14), containing 4 percent dextrose. On newly stored carrots, in normal storage atmosphere, there would be available to the pathogen nearly 4 percent sucrose, approximately 2 percent reducing sugar (25), slightly less polysaccharides, plus the products of hydrolysis as enzymes attack cell wall pectins.

The yeast, C. krusei, has been isolated from the soil and from dried fruits (64). As a food spoilage organism it most frequently occurred on products containing lactic acid, such as milk, fruit juices, sauerkraut, and pickle brine (29). C. krusei was not one of the few yeasts listed as having the ability to hydrolyze pectin (38). The yeast is not known to attack undamaged carrot tissue. The tissue requirements of this fungus would appear not to be met until normal host cell metabolism and structure have been disrupted.

Respiratory metabolism of carrot tissue under anaerobic conditions has received considerable attention (10, 23, 30, 44, 53, 72). Various workers have shown that small amounts of carbon dioxide accumulation may be desirable in storage of fruit (17, 51, 71). Carrots benefited by increases of carbon dioxide, for short periods of time, provided it did not become excessively high (70). Heavy concentrations of carbon dioxide tended to inhibit respiration, due to a narcotic effect on the surface of key respiratory enzymes. Thus, various reports (23, 28, 57) pointed to the inhibition of succinic oxidase and cytochrome oxidase (41) as pyruvate evidently was diverted from the normal tricarboxylic cycle to form fermentation products. Anaerobiosis occurred fairly quickly, in the case of some film packaged fruits and vegetables, as shown by Scott, et. al. (59). In this instance sweet corn in sealed leak-proof plastic bags stored at low temperatures accumulated an atmosphere of high carbon dioxide and almost complete oxygen depletion in 5 days. Miller and Brooks (43) treated sweet corn and peas with carbon dioxide, at low temperature, and found that the process of conversion of sugar to starch was retarded. Miller (44), subsequently showed that a similar treatment on carrots retarded the conversion of sucrose into reducing sugar. The treatment reduced the total acidity and hydrogen ion concentration of carrot tissue, as long as oxygen was present. In the absence of oxygen, however, an increase in hydrogen ion concentration took place. Substantially the same results are reported by Thornton (67). Choudhury(10) measured the respiratory rate of whole carrots and concluded that oxygen did not seem to

be essential for the production of respiratory sugar.

Sometimes the partial or complete exclusion of oxygen resulted in pitting of stored vegetables at low temperatures (49). Cause of pitting seemed to be abnormal respiration resulting in sub-oxidation and formation of toxic substances which affected tissue near the epidermis. In many cases the condition was simply referred to as low temperature breakdown.

Under normal atmospheric conditions the respiratory response of host tissue to infection is usually an increase in respiration rate. This initial reaction may be connected with host tissue defence reaction, and the pattern can be reversed by inhibiting the phosphorylation reaction. Meyer (42) showed that the rate of entry of Phytophthora infestans into potato tissue was greatly speeded up by treatment with ethanol. High humidity and low oxygen tension also contributed to a decline in host tissue metabolic activity, and the fermentation process predominated. Furthermore, ethanol suppressed synthesis of polyphenol oxidase and decreased the resistance of the host (69). Unlike a number of fruits and vegetables studied when stored in nitrogen, carrots have an ethanol-carbon dioxide ratio of nearly one (31). Sugar loss from carrots in nitrogen was found to be entirely accounted for by alcohol and carbon dioxide produced. It was concluded that the Embden-Meyerhof-Parnas glycolytic pathway was operating in carrot sugar breakdown. Traces of acetaldehyde, glycerol, and phosphate esters were found in these carrot tissues. Using black currants, Smith (62) found that in carbon dioxide zymasis with the presence of oxygen, twice as much alcohol as acetaldehyde

formed. When the external concentration of oxygen fell very low there was an accumulation of these two products. In the complete absence of oxygen the proportion of alcohol to acetaldehyde became very high. Some of this alcohol may be metabolized by carrot tissue itself (15) to form organic acids of the tricarboxylic acid cycle.

The above-mentioned artificial conditions generally unfavourable to the host are not without drawbacks to most plant pathogens. Thus, although sealed plastic storage containers encourage the maintenance of high humidity so essential to the development of S. sclerotiorum, these benefits are outweighed by the retarding effects of increased carbon dioxide. A few fungi tolerate high levels of carbon dioxide (7, 68); most are seriously affected at relatively low levels (6, 7). The retarding effect is greatly enhanced at low temperatures (45). Brown (7) showed that germination of fungal spores was slowed in the presence of increased carbon dioxide, and that developing mycelium tended to form swollen cells. A good nutrient supply, however, helped to overcome the retarding effect. Apparently, variations in oxygen levels had no appreciable effect on fungal development.

According to reports of Brown (7) and Toler, et. al. (68) high carbon dioxide build-up, and accumulation of ethyl alcohol seemed most likely to be the main environmental factors responsible for the carrot cup rot occurring following penetration by S. sclerotiorum. This portion of the report, then, is based mainly on the response of S. sclerotiorum to various sugar, alcohol, and carbon dioxide concentrations. Also, it deals

with changes in storage container atmosphere, as well as tissue pH, favourable to the development of C. krusei. It was hoped that the growth response in vitro of S. sclerotiorum, and in vivo of C. krusei, would throw some light on the physiologic nature of carrot cratering which occurred in heavy-duty sealed polyethylene bags stored at low temperature.

MATERIALS AND METHODS

The Fungus

The isolate of S. sclerotiorum used has been described in the section dealing with the pathologic nature of carrot cup rot. It was maintained by mass mycelial transfers on MYA in slant culture tubes. In liquid medium it grew well from sclerotia previously grown on liquid medium. A thick gelatinous mycelial mat formed which was quite unlike the growth produced on solid medium. Sclerotia from fungal growth on solid medium did not grow well when placed in the liquid medium. Periodically, during the course of the study, S. sclerotiorum reaction was tested on Brooks carrots. During this time it maintained its pathogenicity.

Fungal Growth Studies

The growth response of S. sclerotiorum in a nutrient solution involved the determination of dry weight of mycelium produced in liquid culture in 50 ml of medium in 250 ml Erlenmeyer flasks. The flasks and other glassware used in these tests were first soaked overnight in a strong solution of Sparkleen detergent (Fisher Scientific). Then the flasks were scrubbed thoroughly and rinsed in hot tap water followed by distilled water.

The flasks, and other glassware, were filled with demineralized water and let stand 24 hours to remove any detergent clinging to the glass surface.

Seeding of the liquid medium required the use of sclerotial inoculum, owing to growth failure when macerated mycelium was used. Maceration of sclerotia took place in a Waring blendor, the tumbler of which had been filled with methyl alcohol, emptied, flamed, and rinsed several times in sterile water. Sclerotia were lifted from liquid Houston's medium and added to 200 ml of sterile demineralized water. The sclerotia were blended for 6 minutes to produce particles of fairly uniform size. Houston's basic medium was used to provide nutrients for growth of S. sclerotiorum. Glassware used in preparation of the medium was cleaned in the manner described for preparation of growth flasks.

Following are constituents of the basal medium outlined by Houston:

Dextrose	40.0 g
$MgSO_4.7H_2O$	0.3 g
KH_2PO_4	0.3 g
K_2HPO_4	1.2 g
$FeCl_3.6H_2O$	1 ppm
$CuSO_4.5H_2O$	1 ppm
$ZnSO_4.7H_2O$	1 ppm
$MnSO_4.5H_2O$	1 ppm
Demineralized water	1000 ml

The latter 4 minor elements were prepared in stock solution, refrigerated, and added to the medium through sterile

pipettes as required. Stock medium was prepared in 5 litre quantities and refrigerated until required. Fifty ml of medium in each 200 Erlenmeyer flask was autoclaved for 15 minutes at 15 pounds pressure and cooled at room temperature before inoculating. A 2 ml eyedropper cleaned in alcohol and rinsed in sterile water was used for adding inoculum to the medium. Ground sclerotia in a covered sterile beaker were kept in suspension by a mechanical stirring rod. Approximately 1.8 ml of inoculum suspension was added to each growth flask. Flasks were placed on a shake rack at 20° C and inoculum allowed to grow until the fungus had made satisfactory growth in any one treatment. At this point all flasks in each treatment were removed, examined for contamination, and non-contaminated growth filtered for drying and weighing. Weighing containers to handle the 5 replicates in each treatment were small aluminum trays oven-dried at 90° C for 24 hours, and placed in desiccators during cooling prior to weighing. Mycelium adhering to the filter paper was rinsed several times to remove traces of the nutrient solution. Filter papers containing the mycelium were then folded and placed on numbered trays to be oven-dried at 90° C for 24 hours. After drying, they were again placed in desiccators during cooling prior to weighing.

Ethyl Alcohol in Growth Studies

Standard Houston's medium was prepared and autoclaved in 200 ml Erlenmeyer flasks. A 10 percent alcohol stock solution was prepared by adding 95 percent ethyl alcohol to sterile Houston's medium. By means of sterile pipettes the alcohol stock solution was added to each of the Erlenmeyer flasks. Five

replicates were used in each treatment. To each of the flasks containing the Houston's-alcohol solution was added 1.8 ml ground sclerotia suspension. The cotton plug and neck of each flask was tightly wrapped with plastic film to reduce alcohol evaporation. Flasks were placed on a shake rack for growth of inoculum at 20° C. When the fungus in any treatment had made satisfactory growth all flasks in every treatment were removed. After discarding any contaminated flasks mycelium in each treatment was filtered and washed before drying in aluminum trays at 90° C for 24 hours. Following oven-drying, trays containing mycelium and filter paper were cooled in desiccators prior to weighing.

Carbon Dioxide in Growth Studies

For this portion of the study desiccators were used as growth chambers. The volume of air, in each desiccator containing 5 culture plates, was measured by filling with water. For each treatment, 5 culture plates containing MYA were inoculated with a 5 mm plug of S. sclerotiorum grown on MYA. After carefully sealing the lids on the desiccators the air within was evacuated with an aspirator at a suction pressure of 25 pounds per square inch. To each desiccator was added a volume of carbon dioxide estimated to provide approximately the percentage concentration desired. Nitrogen was added to fill the remaining space, equalizing internal pressure and room air pressure. Approximately 24 hours after the start of each experiment, actual oxygen and carbon dioxide readings were

made, using the Haldane gas analyzer. Duplicate readings were made for each treatment. The rubber tip of a medicine dropper served as a cover for the desiccator stop-cock arm, when air was being withdrawn for analysis. The needle of a 10 c.c. hypodermic syringe was inserted through the stop-cock cover, and 10 c.c. of air was drawn into the syringe for each reading. The procedure for analyzing oxygen and carbon dioxide content of air has been outlined in Part 1 of the investigations.

EXPERIMENTAL RESULTS

Concentration of Dextrose for Optimum Growth of *S. sclerotiorum*

S. sclerotiorum was grown in Houston's medium with concentrations of dextrose ranging from 10 g to 140 g per litre. The results (Fig. 9) indicate a gradual increase in growth of the fungus for increasing concentrations of dextrose to 100 g per litre. Further increases in dextrose concentration were followed by a sharp decline in growth. Maximum growth response was established at 100 g dextrose per litre Houston's medium.

Effect of Ethyl Alcohol on Growth of *S. sclerotiorum*

Initial trials indicated that the growth of *S. sclerotiorum* was completely inhibited in Houston's medium containing 4 percent ethyl alcohol. To determine the minimum concentration of alcohol which would fully inhibit growth of the fungus, solutions containing alcohol ranging from 2.4 to 3.4 percent were used. Results obtained are the average of 5 replicates in each treatment. Growth in Houston's medium containing 2.4 percent alcohol was slightly less than growth in Houston's alone (Fig. 10).

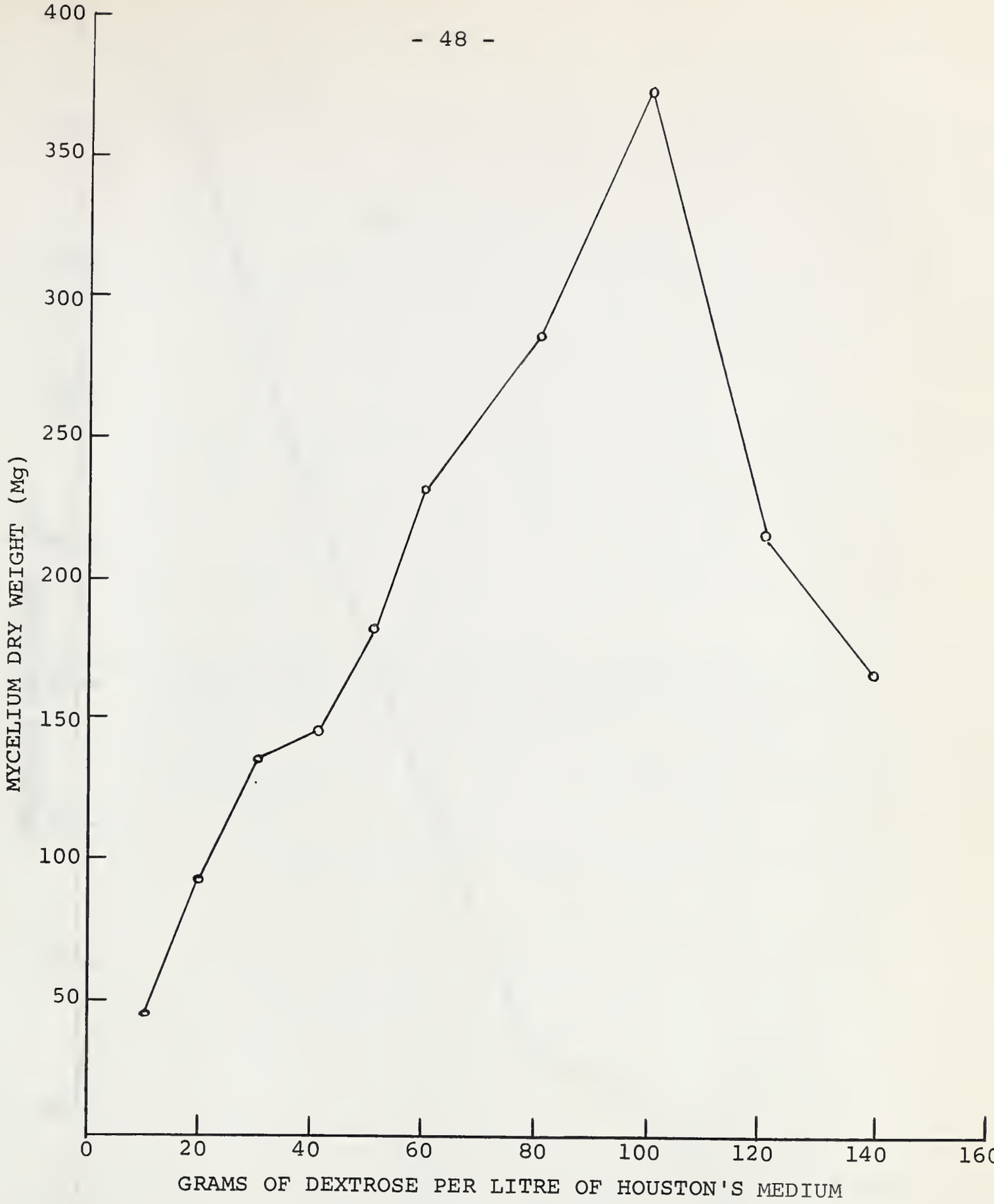

Fig. 9. The effect of different concentrations of dextrose in Houston's medium on the growth of S. sclerotiorum.

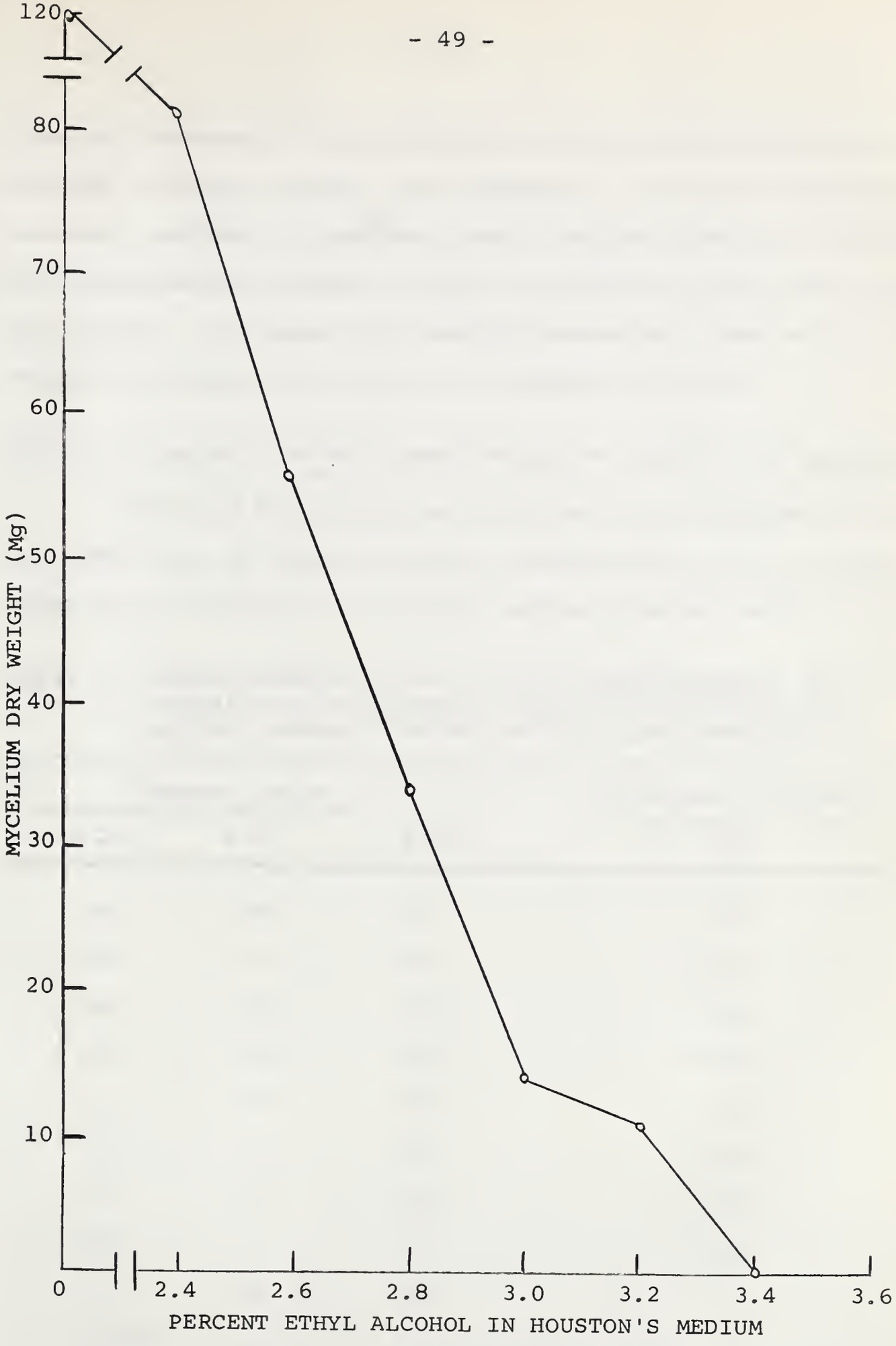

Fig. 10. The effect of different concentrations of alcohol in Houston's medium on the growth of S. sclerotiorum.

However, increasing concentrations of alcohol resulted in a sharp decline in fungal growth. For instance a 0.6 percent increase in alcohol, over the 2.4 percent level, reduced growth by 70 percent. At concentrations between 3.0 and 3.2 percent alcohol the decline was slight. It dropped off sharply thereafter, however, until fungal growth did not occur at 3.4 percent alcohol.

Effect of Carbon Dioxide Concentration on Growth of S. sclerotiorum

Growth of the fungus was inhibited when compared to that in normal air, at carbon dioxide concentrations up to 30 percent. There was no growth at 13 percent carbon dioxide (Table 7), but

Table 7. Radial growth at 27° C of S. sclerotiorum on MYA in equilibrium with gaseous phases having different oxygen, carbon dioxide, and nitrogen combinations.

Gaseous phase			Difference in growth from that in air (%)
% CO_2	% O_2	% N_2	
30	16	54	-100
28	6	66	-100
16	6	78	-100
13	6	81	-100
11	7	82	-90
9	7	84	-84
4	8	88	-43
3	5	92	-23
1	19	80	-2
0.03	21	78.97 (air)	0

the mycelium was not killed. Upon return to air the fungus resumed normal rate of growth within 24 hours. Growth inhibition was sharp, comparing growth in air to growth at various concentrations up to 3 percent carbon dioxide. Inhibition continued, but less sharply, until reaching 9 percent carbon dioxide. At this point fungal growth was less that 50 percent of growth in air. From 9 to 13 percent carbon dioxide, growth decline once again was sharp.

There was stimulation of growth, compared to growth in lower carbon dioxide concentrations, of S. sclerotiorum as carbon dioxide increased from 4 to 8 percent. Such stimulation took place only when mycelial discs for seeding plates were taken from cultures which had been maintained continuously on MYA. These parent cultures could usually be recognized by a dark brown pigmentation of the older mycelium. The response was the same, regardless of whether the seeding inoculum was removed from the discolored plates, or from actively growing mycelium once removed from such plates. When seeding discs were taken from plates of actively growing mycelium obtained from Houston's liquid medium, no stimulation by carbon dioxide occurred.

DISCUSSION

Physiologic investigations showed that the growth of S. sclerotiorum can be inhibited by relatively low concentrations of carbon dioxide, or by ethyl alcohol. Within wide limits increasing sugar concentrations, on the other hand, stimulate growth. Sugar concentration for maximum growth on artificial

medium was two and one half times the sugar content of carrots. The gap would be narrowed, it is supposed, as fungal enzymes release sugars in the process of breaking down cell wall materials.

Ethyl alcohol in low concentration readily inhibited the growth of S. sclerotiorum. When alcohol levels reached 3.4 percent in liquid nutrient medium growth of the fungus stopped. As indicated in Part I, at this concentration of alcohol in carrot tissue, growth of S. sclerotiorum had stopped, but C. krusei was still very active. Assuming that S. sclerotiorum response to alcohol in carrot tissue is similar to its response in a liquid medium, alcohol could halt growth of the fungus, where cup rot is involved.

Carbon dioxide concentrations capable of stopping the growth of S. sclerotiorum at room temperature, were reached after 2 weeks of carrot storage at low temperature. In the light of Lauritzen's (37) report, the fungus would be active for a period of less than two weeks at the beginning of the storage period, since low temperature lessens the concentration of carbon dioxide required to halt growth. As long as increased carbon dioxide was maintained in growth chambers fungal inhibition continued. Likewise, in carrot storage bags growth was halted. However, when bags were opened after a longer storage period, no growth of S. sclerotiorum ensued. Brown (8) has indicated that the macerating enzymes of S. sclerotiorum are capable of functioning for some time independently of the fungus. Eventually, however, they lose their power of penetrating tissue. When this occurs, no further cell breakdown takes place. Bruised carrot

tissue (Table 1) is particularly susceptible to invasion by S. sclerotiorum, even at low temperature.

The stimulating effect of carbon dioxide on S. sclerotiorum was confined to mycelial discs from MYA medium. Poor growth in air following seeding suggests that a "staling" action may have been involved. The fact that mycelial transplants from fresh liquid medium always grew well tends to support this hypothesis. A tentative explanation for the corrective effect of carbon dioxide on "stale" inoculum is that the gas causes a favourable change in pH of the mycelium. At concentrations below 4 percent, carbon dioxide levels were not high enough to effect a noticeable change in pH. At concentrations above 9 percent, carbon dioxide became inhibitory.

Results obtained in these investigations suggest that low storage temperatures alone are insufficient to fully inhibit the activity of S. sclerotiorum or C. krusei. Both organisms are capable of continuing, although slowly, at temperatures close to the freezing point of carrot tissue. It has also been shown that a non-pathogenic organism is capable of destroying the market value of stored carrots, when tissue has been subjected to a combination of undesirable environmental influences.

Accumulation of carbon dioxide alone can precipitate a complete breakdown of carrot tissue, if the storage period is prolonged. However, as mentioned by Tompkins (71) and as shown in the present study, leaks in bags usually occur. This leads to a moderation in carbon dioxide build-up within storage containers. Present packaging materials, when stored at low

temperatures, are likely to develop leaks at seams, ties, and at pressure points.

C. krusei does not require that oxygen be excluded in order to penetrate damaged carrot tissue. Neither is it adversely affected by the competing fungus. In fact S. sclerotiorum, through its ability to lower tissue pH and to penetrate and macerate cell materials, provides a favourable environment for development of C. krusei. Although the latter is not a high alcohol producer, amounts eventually are sufficient to inhibit completely growth of the initial invader. Under the type of storage conditions involved in the present investigations alcohol production, associated with the activity of C. krusei, could fully restrain S. sclerotiorum. However, build-up of carbon dioxide, in most cases, probably was the original controlling influence.

Assuming that moderate concentrations of carbon dioxide have no deleterious effect on carrots stored at low temperatures for prolonged periods, bag perforations, natural leaks, or films of greater semi-permeability would be desirable. Thus, it may be theoretically possible to hold S. sclerotiorum in check by careful manipulation of bag atmosphere. Unfortunately, two obstacles would still have to be overcome to prevent cup rot from occurring. The first is the lag period in cooling carrots in bulk storage quickly enough to halt initial invasion of tissue, as well as the delay in natural build-up of carbon dioxide. Secondly, surface disinfestation to eradicate microorganisms must be done with considerable care and may be time-consuming. It has previously been mentioned that lowering the relative

humidity during the entire storage period, to circumvent the development of S. sclerotiorum, may result in poorer carrot quality. Decreasing the amount of inoculum originally present on the surface of newly harvested carrots, and pre-drying after washing, may be preferable.

Artificially induced anaerobiosis at the time carrots are packaged could eliminate S. sclerotiorum immediately. Under such conditions, however, carrot quality can deteriorate in a short while. To prevent this happening, high carbon dioxide concentrations could be applied to bags for a week or less at the start of the storage period. This would serve to prevent the initial penetration and development of S. sclerotiorum. Release of a portion of the carbon dioxide pressure would prevent gas damage to carrot tissue, and the fungus could still be held in check. Without penetration by S. sclerotiorum, and by minimizing bruising during handling of carrots, cup rot could be prevented since C. krusei would lack an available food supply.

GENERAL DISCUSSION AND CONCLUSIONS

The hypothesis that cup rot of carrots stored in closed polyethylene bags was incited primarily by S. sclerotiorum followed by C. krusei proved to be correct. The sequential appearance of the two fungi was influenced by a set of conditions peculiar to the closed storage environment.

Presumably, carrots are infected by S. sclerotiorum in the field where the fungus persists in the soil. This pathogen, through its enzymic action, macerates the host tissue thus releasing the cell contents. Meanwhile, the acidity of the carrot tissue is increased considerably in the area of infection. The maceration of carrot tissue and pH changes would ordinarily continue until the entire carrot is destroyed. This, in fact, is what happens when carrots are stored under aerobic conditions and Sclerotinia watery rot develops.

The air in closed polyethylene bags containing carrots increases sharply in carbon dioxide concentration while the oxygen content is reduced. The carbon dioxide content rises to a point where it inhibits the growth of S. sclerotiorum and at the same time favours the development of C. krusei which is present on carrots. Two other factors which have developed in the diseased carrot tissue favour the growth of the yeast. The tissue is sufficiently macerated to make nutrients readily available to the saprophyte, and the high acidity forms a natural environment. As the yeast develops rapidly it produces alcohol through its fermentation process. The alcohol content can rise sufficiently

to be inhibitory to the growth of S. sclerotiorum. Thus the combined effects of carbon dioxide and alcohol tend to eliminate S. sclerotiorum while the available nutrients, the high carbon dioxide, and the acid content tend to favour C. krusei.

The end result is that the yeast dominates and utilizes the tissue macerated by S. sclerotiorum. However, once that tissue is used up, the healthy portion of the carrot is left intact, since C. krusei is not pathogenic. The resulting symptom is, therefore, a cup-shaped type of rot.

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